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We compared three methods for detecting enteropathogens in 416 children with diarrhea: (i) examination of 10 lactose-fermenting and all non-lactose-fermenting Escherichia coli (colony blots); (ii) examination of 300 colonies (replicate blots); and (iii) determination of the total bacterial growth of stools (stool blots). All specimens were spotted onto Whatman 541 filters and hybridized with specific radiolabeled DNA probes. [illegible] were detected in 38 [illegible] by examining [illegible] replicate blots,

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Examination of Colonies and Stool Blots for Detection of Enteropathogens by DNA Hybridization with Eight DNA Probes

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We compared three methods for detecting enteropathogens in 416 children with diarrhea: (i) examination of 10 lactose-fermenting and all non-lactose-fermenting *Escherichia coli* (colony blots); (ii) examination of 300 colonies (replicate blots); and (iii) determination of the total bacterial growth of stools (stool blots). All specimens were spotted onto Whatman 541 filters and hybridized with specific radiolabeled DNA probes. Enterotoxigenic *E. coli* was detected in 38 patients by examining colony blots, in 52 patients by examining replicate blots, and in 45 patients by examining stool blots. Enteropathogenic *E. coli* adhesin factor was detected in 12 patients by colony blots, in 25 patients by replicate blots, and in 16 patients by stool blots. *E. coli* that hybridized with the enterohemorrhagic *E. coli* probe was detected in 2 patients by colony blots, in 11 patients by replicate blots, and in 0 patients by stool blots. Shiga-like toxin-producing *E. coli* was detected in 0 patients by colony blots, in 12 patients by replicate blots, and in 0 patients by stool blots. *Shigella* spp. were identified by standard bacteriological methods in 82 patients, and enteroinvasive *E. coli* was identified by colony blots in 11 patients (total, 93), by replicate blots in 56 patients, and by stool blots in 35 patients. Of 82 culture-confirmed *Shigella* infections, 45 were identified by examining replicate blots with the 17-kilobase-pair probe and 36 were identified by examination with the Ipa probe ($P < 0.05$). Examining replicate blots with specific probes identified more enterotoxigenic *E. coli* ($P < 0.005$), enteropathogenic *E. coli* adhesin factor-producing *E. coli* ($P < 0.001$), and Shiga-like toxin-producing *E. coli* ($P < 0.005$) infections than examining colony blots. More *Shigella* and enteroinvasive *E. coli* infections were identified by standard bacteriological methods and examining colony blots with a specific probe than by examining replicate and stool blots ($P < 0.001$).

Pathogenic determinants have been identified in *Escherichia coli* isolated from patients with diarrhea. These include enterotoxin production, enteroinvasion, localized enteroadherence, and Shiga-like toxin (SLT) or verocytotoxin (VT) production (5). DNA probes to identify genes coding for these determinants have been constructed and used to study the etiology of diarrheal disease in various parts of the developing world (2, 4, 12, 13, 20, 23, 24).

Individual colonies and the total bacterial growth of stools have been examined with specific probes to detect enterotoxigenic *E. coli* (ETEC) (3, 12), enteroinvasive *E. coli* (EIEC) (19, 23), enteropathogenic *E. coli* adhesin factor-producing *E. coli* (EAFEC) (3, 14), and *Shigella* spp. (19, 23). Enterohemorrhagic *E. coli* (EHEC) O157:H7 contains a 60-megadalton plasmid required for expression of a new fimbrial antigen adhesion to epithelial cells (6). A cryptic fragment of this plasmid has been used to identify EHEC and has been referred as the EHEC probe (8).

Examining the total bacterial growth of stools identified fewer ETEC, EAFEC, EIEC, and *Shigella* infections than examining individual colonies (2, 4, 24). In a study of patients with hemorrhagic colitis in Great Britain, EHEC was identified in 39% of patients by examining several hundred colonies from each patient for hybridization with the VT I and VT II probes (21). Between <1% and 100% of the colonies examined from each patient were identified as EHEC. To determine the optimal method for identifying diarrheogenic *E. coli* infections, we compared the standard colony blot method with replicate blot and stool blot hybridization methods.

A 17-kilobase (kb) *Eco*RI fragment of the 140-megadalton virulence plasmid of *Shigella flexneri* serotype 5, pWR100, has been used to identify EIEC and *Shigella* spp. (20, 23). Recently, genes coding for the invasion plasmid-encoded antigens have been cloned (1) and a probe coding primarily for invasion plasmid antigen *a* (Ipa) has been used to identify EIEC and *Shigella* spp. (25). We compared the Ipa probe with the 17-kb *Eco*RI probe to identify *Shigella* spp. and EIEC by examining 300 colonies and the total bacterial growth of stools.

MATERIALS AND METHODS

Specimens. From July to December 1987, stools were collected from children under 5 years old with diarrhea who had not received antibiotics in the previous 2 weeks and were seen at the outpatient department of Children's Hospital, Bangkok, Thailand. Diarrhea was defined as three or more loose stools that assumed the shape of the container in 24 h for less than 72 h.

Bacteriology. Stools were examined for bacterial enteric pathogens as previously described (23). *Shigella* spp. and *E. coli* were identified by standard biochemical tests (8). *Shigella* spp. were tested for agglutination in specific antisera (Difco Laboratories, Detroit, Mich.). *E. coli* isolates were saved on nutrient agar slants.

Processing of specimens for DNA hybridization assays. For colony blots, 10 lactose-fermenting (Lac^+) and all non-lactose-fermenting (Lac^-) *E. coli* from each child were inoculated onto MacConkey agar plates, incubated at 37°C for 16 h, plated on eight additional MacConkey plates with

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replicate colony transfer pads (FMC Bioproducts, Rockland, Maine), and incubated at 37°C for 16 h. Colonies were transferred from MacConkey agar to filters (no. 541; Whatman, Inc., Clifton, N.J.) by pressing the filters evenly over the colonies. Filters were subsequently placed on Whatman no. 3 paper saturated with 0.5 N NaOH–1.5 M NaCl, steamed for 3 min in an autoclave, immersed in 1 M Tris–2 M NaCl (pH 7.4) for 4 min, and air dried (10).

For replicate blots, 0.1 ml of 10-fold serial dilutions of 1 g of stool vortexed in 2 ml of sterile phosphate-buffered saline was spread on MacConkey agar with a sterile glass triangle and incubated at 37°C overnight. Plates containing approximately 300 well-separated colonies were replica plated onto eight additional MacConkey plates and incubated at 37°C for 16 h. Colonies on these plates were transferred to Whatman 541 filters and processed as described above (10).

For stool blots, stools were inoculated on a MacConkey plate (1 by 1 cm area) and the total bacterial growth was replica plated onto eight additional MacConkey plates. The total bacterial growth from each plate was transferred to Whatman 541 filters and processed as previously described (10).

DNA probes. Plasmid DNA was isolated from *E. coli* K-12 containing pEWD299 (LT), pCVD419 (EHEC), pJN37-19 (SLT I), pNN110-18 (SLT II), pRM17 (EIEC), pW22 (Ipa), and pMAR22 (EAF) as described by So et al. (22) and digested with appropriate restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.) under conditions specified by the manufacturer. A synthetic 23-mer oligonucleotide probe to detect genes coding for STA was constructed with a DNA synthesizer (Applied Biosystems, Foster City, Calif.) as previously described (4). The LT probe consisted of a 850-base *Hin*cII digestion fragment of pEWD299 (12), the EHEC probe was a 3.4-kb *Hin*dIII fragment of pCVD419 (9), the EIEC probe was a 17-kb *Eco*RI digestion fragment of pRM17 (20), and the Ipa probe was a 1,750-base *Eco*RI digestion fragment of pW22 (1). The SLT I probe was a *Bam*HI 1,142-base fragment of pJN37-19, and the SLT II DNA probe was a *Sma*I-*Pst*I 842-base fragment of pNN110-18 (15). The EAF probe was a *Bam*HI-*Sal*I 1-kb digestion fragment of pMAR22 (14). The appropriate DNA fragments were separated by polyacrylamide gel electrophoresis, electroeluted from the gel, and labeled in vitro with [α-^{32}P]deoxynucleotide triphosphate (Dupont, NEN Research Products, Boston, Mass.) by nick translation (11). The ST oligonucleotide probe was 5′ end labeled with [γ-^{32}P]deoxynucleotide triphosphate (11).

DNA hybridization. Filters examined with the polynucleotide-cloned probes were incubated in the following hybridization solution: 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 sodium citrate), 0.1% sodium dodecyl sulfate, 1 mM EDTA, and Denhardt solution (0.02% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.] [molecular weight, 400,000], 0.02% polyvinylpyrrolidone [molecular weight, 360,000], 0.2% bovine serum albumin). The filters were then transferred to fresh hybridization solution containing 10^6 cpm of heat-denatured DNA probe and 75 μg of sheared heat-denatured calf thymus DNA per ml and incubated at 37°C overnight. Hybridization with the γ-^{32}P-labeled ST oligonucleotide probe was performed at 50°C overnight in 6× SSC–1% bovine serum albumin–1% polyvinylpyrrolidone–1 mM EDTA with 10^7 cpm of oligomer probe per ml.

The filters examined with the DNA polynucleotide probes were washed twice in 5× SSC–0.1% sodium dodecyl sulfate for 45 min at 65°C, rinsed in 2× SSC at 22°C, and air dried.

TABLE 1. Identification of ETEC and EAFEC infections in 416 children with diarrhea by three methods

Organism	No. of infections identified by:		
	Colony blot	Replicate blot	Stool blot
ETEC	38	52	45
EAFEC	12	25	16

Filters examined with the γ-^{32}P-labeled ST oligonucleotide probe were washed in 6× SSC, three times for 30 min each at 50°C, and air dried. Filters were exposed to X-Omat-R X-ray film (Eastman Kodak Co., Rochester, N.Y.) with a single Cronex Lightning-Plus intensification screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) for 24 h at −70°C. Films were developed according to the instructions of the manufacturer. *E. coli* strains that hybridized with the SLT I, SLT II, or EHEC probe were tested for VT production in the Vero cell assay (7), *E. coli* strains that hybridized with the *E. coli* adhesin factor (EAF) probe were tested for mannose-resistant adherence to HeLa cells as described by Scaletsky et al. (17), *E. coli* strains that hybridized with the 17-kb or Ipa *Shigella*-EIEC probe were tested by the Sereny test (18), and *E. coli* strains that hybridized with the LT and ST probes were tested for enterotoxin production (3, 16). No attempt was made to recover isolates that hybridized with DNA probes in replicate blots.

Statistical analysis was performed with the McNemar test for matched-pair analysis.

RESULTS

Comparison of colony, replicate, and stool blots with specific DNA probes. (i) ETEC. Among 416 children with diarrhea, 7 were infected with *E. coli* colonies that hybridized with both the LT and ST probes, 24 were infected with *E. coli* colonies that hybridized with only the LT probe, and 7 were infected with *E. coli* colonies that hybridized with only the ST probe. All of the *E. coli* colonies that hybridized with the enterotoxin gene probes produced enterotoxin(s), as determined by Y-1 adrenal and suckling mouse assays. Colonies that hybridized with the enterotoxin gene probes were identified in 38 children by testing 10 *E. coli* colonies (colony blots), in 52 children by testing 300 colonies (replicate blots) and in 45 children by testing stool blots (Table 1). ETEC was identified in 61 (15%) of 416 children by any method. Among 38 children with ETEC, examining replicate blots with the enterotoxin gene probes had a sensitivity of 92%, a specificity of 96%, a PPV of 67%, and a NPV of 99%. Examining stool blots with the enterotoxin gene probes had a sensitivity of 82%, a specificity of 96%, a PPV of 69%, and a NPV of 98%. The mean percentage of 300 colonies that hybridized with both the LT and ST probes was 93% (95% confidence limits, 80 to 100%), the percentage that hybridized with only the LT probe was 33% (95% confidence limits, 9 to 47%), and the percentage that hybridized with only the ST probe was 57% (95% confidence limits, 28 to 86%).

(ii) EAFEC. *E. coli* strains that hybridized with the EAF probe and adhered to HeLa cells in a localized adherent pattern were isolated from 12 children with diarrhea. EAFEC was identified by colony blots in 12 children, by replicate blots in 25 children, and by stool blots in 16 children (Table 1). EAFEC infections were identified in 27 (6%) of 416 children with diarrhea by any method. The mean percentage of 300 colonies that hybridized with the EAF probe was 71% (95% confidence limits, 54 to 88%). Among

TABLE 2. Identification of 82 *Shigella* and 11 EIEC infections by examining replicate and stool blots with the 17-kb and Ipa radiolabeled probes[a]

Organism	No. of infections identified by:				
	Standard methods	Replicate blot		Stool blot	
		17-kb	Ipa	17-kb	Ipa
Shigella[b]	82	45	36	28	24
EIEC[c]	11	5	6	7	6

[a] The 17-kb probe is the 17-kb *Eco*RI fragment of pRM17; the Ipa probe is the 1,750-base *Eco*RI fragment of pW22.
[b] Identified by standard bacteriological methods.
[c] Identified by testing 10 lactose-fermenting and all non-lactose-fermenting *E. coli* for hybridization with the radiolabeled 17-kb probe and confirming probe-positive isolates by the Sereny test (18).

12 children with EAFEC, replicate blots examined with the EAF probe had a sensitivity of 83%, a specificity of 96%, a PPV of 40%, and a NPV of 99%. Examining stool blots with the EAF probe had a sensitivity of 83%, a specificity of 98%, a PPV of 62%, and a NPV of 99%.

(iii) *E. coli* that hybridized with the EHEC probe. *E. coli* that hybridized with the EHEC probe was identified by colony blots in 2 children, by replicate blots in 11 children, and by stool blots in 0 children. Infections with *E. coli* that hybridized with the EHEC probe were identified in 13 (4%) of 416 children with diarrhea by any method. Replicate blots from three children hybridized with the EHEC probe and SLT probes (SLT I and II, one child; SLT I only, one child; and SLT II only, one child). Eleven *E. coli* colonies that hybridized with the EHEC probe did not hybridize with the SLT probes or product VT. The mean percentage of 300 colonies that hybridized with the EHEC probe was 13% (95% confidence limits, 2 to 27%).

(iv) SLT-producing *E. coli*. None of the colony blots, 12 (2%) of the replicate blots, and none of the stool blots from children with diarrhea hybridized with the SLT probes. Five replicate blots hybridized with the SLT I probe, six hybridized with the SLT II probe, and one hybridized with both SLT probes. Of 300 colonies, a mean of 3% (95% confidence limits, 1 to 5%) hybridized with the SLT probes.

(v) *Shigella* spp. and EIEC. *Shigella* spp. were isolated from 82 children with diarrhea by standard bacteriological methods. Of these 82 culture-confirmed *Shigella* infections, 45 were identified by examining replicate blots and 28 were identified by examining stool blots with the 17-kb *Eco*RI probe ($P < 0.01$) (Table 2). From the same cases of shigellosis, 36 replicate blots and 24 stool blots hybridized with the Ipa probe ($P < 0.05$). More *Shigella* infections were identified by examining replicate blots with the 17-kb probe than with the Ipa probe (45 versus 36; $P < 0.05$); however, the difference between examining stool blots with the 17-kb probe versus examining blots with the Ipa probe (28 versus 24 infections identified) was not statistically significant. Among 82 patients with *Shigella* infection, examining replicate blots with the 17-kb and Ipa probes had sensitivities of 55 and 34%, specificities of 98 and 100%, PPVs of 83 and 82%, and NPVs of 91 and 89%. Examining stool blots from the same patients with the 17-kb and Ipa probes had sensitivities of 34 and 30%, specificities of 100 and 100%, PPVs of 100 and 96%, and NPVs of 86 and 87%.

EIEC was isolated from 11 children with diarrhea by testing *E. coli* for hybridization with the 17-kb probe and testing probe-positive isolates in the Sereny test. Nine children were infected with Lac$^+$ and two with Lac$^-$ EIEC.

TABLE 3. Identification of *Shigella* spp. and enterovirulent *E. coli* in 416 children with diarrhea by three methods

Organism	No. (%) of infections identified by[a]:		
	Colony blots	Replicate blots	Stool blots
ETEC	38 (9)	52 (12.5)	45 (11)
EAFEC	12 (3)	25 (6)	16 (4)
Shigella spp.[b]-EIEC[c]	93 (22)	56 (13)	35 (8)
SLTEC[d]	0	12 (3)	0
EHEC[e]	2 (<1)	11 (3)	0

[a] For colony and replicate blots, P is <0.005 for data for ETEC and SLT-producing *E. coli* and <0.001 for data for EAFEC and *Shigella* spp.-EIEC; for replicate and stool blots, P is <0.005 for data for *Shigella* spp.-EIEC, SLT-producing *E. coli*, and EHEC. All other differences are not significant.
[b] Identified by standard bacteriological methods (9).
[c] Identified with the 17-kb DNA probe.
[d] SLTEC, SLT-producing *E. coli*.
[e] EHEC identified with the EHEC probe.

One Lac$^-$ EIEC infection was detected by the 17-kb and the Ipa probes in both replicate and stool blots, while the other Lac$^-$ EIEC infection was not detected in replicate or stool blots with either probe. There was no significant difference between identifying EIEC in replicate blots (five and six EIEC identifications) and stool blots (seven and six EIEC identifications) with the 17-kb and Ipa probes (Table 2).

A total of 82 *Shigella* infections were identified by standard bacteriological methods, and 11 EIEC infections were identified by colony blots (total, 93), 56 were identified by replicate blots, and 35 were identified by stool blots in 416 children with diarrhea. In six patients, replicate blot hybridized with the 17-kb and Ipa probes, but *Shigella* and EIEC were not isolated. The mean percentage of 300 colonies that hybridized with the 17-kb probe in *Shigella* infections was 43% (95% confidence limits, 8 to 86%), and the percentage in EIEC infections was 47% (95% confidence limits, 8 to 86%). Among 11 children with EIEC infections, examining replicate blots with the 17-kb and Ipa probes had sensitivities of 45 and 50%, specificities of 98 and 98%, PPVs of 36 and 38%, and NPVs of 99 and 99%. Examining stool blots from the same patients with the 17-kb and Ipa probes had sensitivities of 64 and 54%, specificities of 100 and 100%, PPVs of 100 and 86%, and NPVs of 99 and 99%.

The identification of *E. coli* and *Shigella* infections by standard bacteriological methods and by examining colony, replicate, and stool blots is summarized in Table 3.

DISCUSSION

More *E. coli* infections were identified by examining replicate blots than by examining colony blots with specific DNA probes. Examining replicate blots identified more *E. coli* and *Shigella* infections than examining stool blots, although this was not significant with ETEC and EAFEC infections. Since stool blots contain 10^8 to 10^9 bacteria, whereas replicate blots contain approximately 300 well-separated colonies, nonhomologous DNA or bacterial wall remnants appear to interfere with the access of specific probes to target cell DNA. Lac$^-$ *Shigella* and Lac$^-$ EIEC may be overgrown by faster growing Lac$^+$ *E. coli* in stool blots. Bacterial pathogens may not be evenly distributed in specimens, e.g., *Shigella* spp. and possibly Lac$^-$ EIEC, and the infected areas may not be cultured in the preparation of stool blots. Examining replicate and stool blots was less sensitive in identifying *Shigella* infections than standard bacteriological methods (20, 24) and should not be used to

identify *Shigella* spp. and EIEC in clinical specimens from patients with diarrhea.

The percentage of 300 colonies that hybridized with DNA probes for genes coding for pathogenic determinants was low in some children with diarrhea. This method may not always identify bacteria that are etiologically important. LT ETEC identified in 33% of 300 colonies have not been associated with diarrhea disease in children less than 5 years old (P. Echeverria, D. N. Taylor, U. Lexomboon, M. Bhaibulaya, N. R. Blacklow, K. Tamura, and R. Sakazaki, J. Infect. Dis., in press).

The 11 *E. coli* colonies that hybridized with the EHEC probe did not hybridize with the SLT I and SLT II probes or produce VT. Seriwatana et al. (19) previously described *E. coli* isolates that hybridized with the EHEC probe that did not produce VT. Although the EHEC probe hybridized with most EHEC isolated in Canada, Germany, the United States, and Chile, this probe hybridizes with *E. coli* that do not produce VT. It is possible that the *E. coli* isolates that hybridized with the EHEC probe previously contained phage-encoded genes for SLT.

DNA probes have been extremely useful in identifying genes coding for enterovirulent determinants in large numbers of *E. coli*. Examining 300 colonies in replicate blots increased the identification of diarrheogenic *E. coli*. How useful this will be in defining the relative importance of bacterial pathogens in patients with diarrheal disease remains to be determined.

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